

## Nutrient Levels Do Not Affect Male Gametophyte Induction by Antheridiogen in *Ceratopteris richardii*

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**ABSTRACT.**—In the homosporous fern *Ceratopteris richardii*, sex is not determined chromosomally. Rather, hermaphroditic gametophytes produce a hormone called antheridiogen, which induces maleness in undifferentiated gametophytes. The percentage of males increases with increasing density of gametophytes, presumably due to the cumulative effect of antheridiogen from multiple hermaphrodites.

Some have argued that antheridiogen lessens competition between gametophytes. Such competition is expected to be most intense between hermaphrodites given that they support zygote, embryo, and sporophyte growth. Therefore, it is predicted that at lower nutrient levels, the effect of antheridiogen in inducing male gametophytes is greater than at higher nutrient levels.

To test this hypothesis, *C. richardii* spores were sown over a range of densities (0.52/cm<sup>2</sup> to 5.2/cm<sup>2</sup>) in four nutrient-level treatments (100, 50, 25, 12.5 percent of full-strength nutrient agar). Gametophytes were grown for four weeks at 28 degrees Celsius with a photoperiod of 14 L: 10 D. An ANCOVA found an overall positive relationship between gametophyte density and percentage of male gametophytes. However, the relationship between gametophyte density and percentage of male gametophytes did not differ among nutrient levels. Nutrient levels had no effect on the rate of male induction by antheridiogen. A post-hoc power analysis showed that the experimental power was 97%.

**KEY WORDS.**—*Ceratopteris*, sex determination, gametophyte, antheridiogen

The gender of sexually dimorphic plant species may be determined genetically, environmentally, or by a combination of the two (Lloyd and Bawa, 1984; Meagher, 1988). If sex in a species is determined by environmental factors, males should be more common in low-resource environments (Schlessman, 1988). In flowering plants, this occurrence has been explained by a higher cost of producing ovules, seeds, and fruits versus producing pollen (Lovett Doust and Harper, 1980; Lovett Doust and Lovett Doust, 1983). A similar tendency is seen in seedless vascular plants, in which the female/hermaphroditic gametophytes endure a higher reproductive cost because they are responsible for producing the egg, the zygote, and the embryo, as well as supporting the developing sporophyte (Sakamaki and Ino, 1999). Therefore, if sex determination in seedless vascular plants is analogous to flowering plants, then resource availability in the environment may be expected to have a direct effect on sex determination of gametophytes (Sakamaki and Ino, 1999).

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Specifically, males should be more common in environments with low resource levels.

Sex determination in the homosporous fern *Ceratopteris richardii* Brongn. is plastic. Spores develop into gametophytes that are either hermaphroditic, containing both archegonia and antheridia, or exclusively male, containing only antheridia (Banks, 1997). Developing and mature hermaphrodites produce a gibberellin-like hormone (Warne and Hickok, 1989) called antheridiogen ( $A_{CE}$  in *C. richardii*; Banks, 1997).  $A_{CE}$  induces maleness in developing gametophytes (Banks, 1997), provided that exposure occurs between the third and the sixth day after spore inoculation (Banks *et al.*, 1993; Eberle *et al.*, 1995). In the absence of  $A_{CE}$ , spores develop into hermaphrodites (Banks, 1997). This model of hormonally determined sex is not exclusive to *C. richardii* but is fairly common to many homosporous ferns (Haig and Westoby, 1988).

Multiple experiments have demonstrated that spore density has an effect on sex determination in *C. richardii* (Warne and Lloyd, 1987; Hickok *et al.*, 1995; Spiro and Knisely, 2008), as well as in another homosporous fern *Osmunda cinnamomea* L. (Huang *et al.*, 2004). More specifically, the sex ratio is skewed toward males at high gametophyte densities. One explanation for this observation is that at higher densities, undifferentiated gametophytes are clustered closer together within the neighborhood of multiple hermaphrodites and are thus exposed to the cumulative effects of  $A_{CE}$ . This hypothesis has indirect, empirical support in that experimentally elevating exogenous antheridiogen leads to male-skewed sex ratios in *C. richardii* (Warne and Hickok, 1991) and other species of homosporous ferns (Cousens and Horner, 1970; Stevens and Werth, 1999; Huang *et al.*, 2004). However, there is a maximum concentration of antheridiogen above which increasing the amount of the pheromone has no additional effect on sex ratios (Cousens and Horner, 1970; Stevens and Werth, 1999; Huang *et al.*, 2004).

An alternative hypothesis for the skewed sex ratios with increasing density relates to the resource cost of being male versus hermaphroditic. If the sex of the gametophytes is based on the resource state of the environment, or if the resource state alters the effects of exogenous  $A_{CE}$ , then the same pattern of gametophyte density and sex ratios would be expected: a higher ratio of males in low nutrient level culture and a lower ratio of males at higher nutrient levels for similar gametophyte densities. In high density cultures, resource levels per gametophyte are expected to be lower, while in low density cultures, resource levels per gametophyte are expected to be higher. The objective of this experiment is to determine whether the proportion of hermaphrodites in a culture of a given density may be altered by the level of nutrients.

#### MATERIALS AND METHODS

*Ceratopteris richardii* Petri dish cultures were established on nutrient agar following Hickok and Warne (2004) using wild type *C. richardii* spores and powdered media obtained from Carolina Biological Supply Company.

Four experimental treatments were established using serial dilutions; 100% nutrient level, 50% nutrient level, 25% nutrient level, and 12.5% nutrient level. These nutrient levels are relative to the maximum level found in the powdered media. For a detailed list of nutrient components in media see Hickok and Warne (2004). Spores were sown on 35mm  $\times$  10mm Petri dishes containing the four nutrient treatments. Spore densities ranged from five to 50 spores per Petri dish and increased at increments of five spores. This yielded an overall density of 0.52 spores/cm<sup>2</sup>–5.2 spores/cm<sup>2</sup>. Each of the four nutrient-level treatments contained 40 Petri dishes (four at each of the 10 spore densities) for a total of 160 dishes.

Spores were incubated at  $29 \pm 3^\circ\text{C}$  under grow lights (24 W/m<sup>2</sup>) for 25 days following a 14 hours day/10 hours night cycle. At that time, determination of the sex of a high proportion of gametophytes was possible. The number of hermaphrodites, males, ungerminated spores, and spores of indeterminate sex were recorded. Hermaphrodites contain archegonia, antheridia, and a notch meristem, giving them a mitten-shaped appearance, whereas males contain antheridia, and are essentially oval (Banks, 1997). The shape of the gametophyte was the primary characteristic used for identification. The percentage of hermaphrodites in each Petri dish was determined by dividing the total number of hermaphrodites by the sum of the males and the hermaphrodites.

To determine if nutrient levels affected the proportion of hermaphrodites, an Analysis of Covariance (ANCOVA) was performed using SYSTAT (Wilkins, 2002). This analysis determined 1) whether the density of gametophytes was related to the proportion of hermaphroditic gametophytes and 2) whether the different nutrient level treatments had an effect on this relationship. The ANCOVA model included nutrient level as a categorical variable and density of gametophytes as a covariate. After testing for the homogeneity of slopes (i.e., the nutrient level\*density of gametophytes interaction), the mean squares and degrees of freedom were excluded from the analysis (pooled into the error term) if  $p > 0.05$ .

With the lack of significance for the ANCOVA, a Power Analysis using G\*Power (Faul *et al.*, 1996) was performed to assess the overall power of the experiment, as well as the probability of making a type II error ( $\beta$ ). A type II error is one in which no effect is detected when an effect really exists (Winer *et al.*, 1991). The effect size necessary for power calculations followed Winer *et al.* (1991).

## RESULTS

Severe agar desiccation in two Petri dishes made it impossible to determine the sex of the gametophytes. As a result, 158 Petri dishes were used in the statistical analysis.

Ninety-nine percent of the gametophytes were identified as either males or hermaphrodites. The slopes of the covariate interactions (nutrient level\*den-

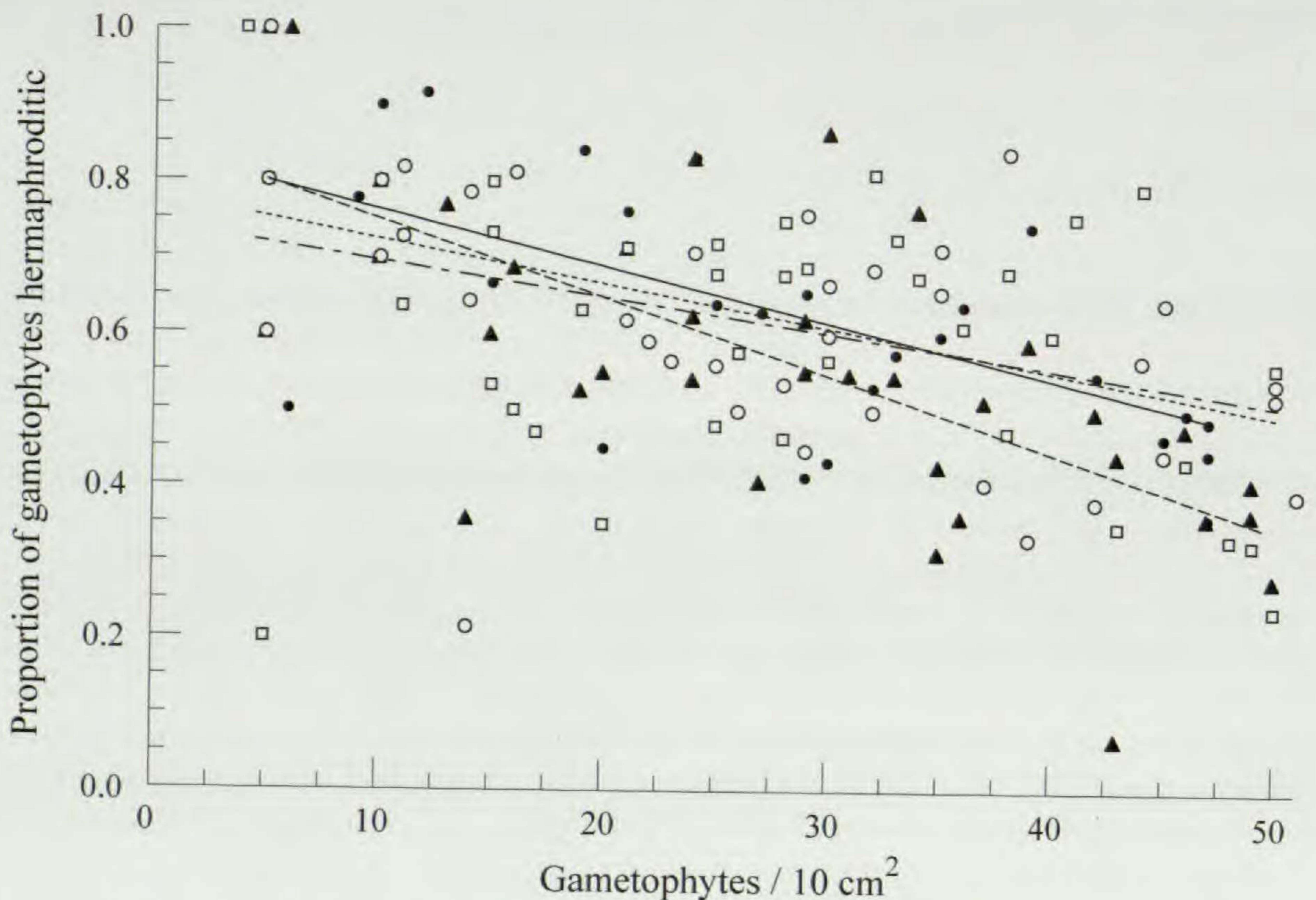


FIG. 1. Relationship between gametophyte density and the proportion of gametophytes hermaphroditic for each of the four nutrient treatments. Closed circle, solid line = 100% nutrient level; open square, dotted line = 50% nutrient level; open circle, dot-dashed line = 25% nutrient level; closed triangle, dashed line = 12.5% nutrient level.

sity of gametophytes) were homogeneous ( $F_{3,150} = 1.932$ ;  $p = 0.127$ ). Therefore, the final analysis did not include this interaction term.

There was an overall relationship between the density of gametophytes and the proportion of hermaphroditic gametophytes (Figure 1;  $p < 0.001$ , Adjusted  $R^2 = 0.353$ ), in which the proportion of hermaphrodites declined with increasing gametophyte density. There was no effect of nutrient level on this relationship ( $F_{3,150} = 1.113$ ,  $p = 0.346$ ).

The statistical power to assess whether nutrient levels affected the relationship between the density of gametophytes and the proportion of hermaphroditic gametophytes required the calculation of an effect size. Effect size was determined to be 0.357 following Winer (1991). The probability of making a type II error,  $\beta$ , was determined to be less than 3%, making the power of this test greater than 97%.

#### DISCUSSION

Similar to previous studies of *Ceratopteris richardii* (Warne and Lloyd, 1987; Hickok *et al.*, 1995; Spiro and Knisely, 2008), this experiment demonstrated a negative relationship between gametophyte density and the proportion of

hermaphrodites that developed. Though this relationship was highly significant ( $p < 0.001$ ), the adjusted  $R^2$  was 0.353, indicating that only 35.3% of the variation in the proportion of hermaphrodites is explained by the variation in gametophyte density. The large amount of unexplained variation (64.7%) indicates that factors other than the presence or absence of  $A_{CE}$  likely influence sex determination in this species. Indeed, hermaphrodites have been shown to develop from young *C. richardii* gametophytes even in the presence of substantial antheridiogen (Warne and Hickok, 1991) or hermaphrodites (Sayers and Hamilton, 1995).

Other factors have been shown to override the effect of  $A_{CE}$  in *C. richardii*. Light quality, biased toward red light, suppresses male development in favor of hermaphroditic development (Kamachi *et al.*, 2007). Smaller spores tend to germinate later than larger spores, with the gametophytes of smaller spores tending to grow more slowly and to develop into males (Sayers and Hamilton, 1995).

While nutrients have been shown to affect sex ratios in at least one other fern, *Dryopteris filix-mas* (L.) Schott. (Korpelainen, 1994), no such effect was shown here for *C. richardii*. The nutrient levels used in this experiment did not alter the effectiveness of  $A_{CE}$ . Similar proportions of hermaphrodites were observed in cultures with similar gametophyte densities regardless of nutrient levels. The experimental power of this experiment was high ( $> 0.97$ ) and therefore the probability of incorrectly concluding that nutrients have no effect on the proportion of hermaphrodites at similar densities is quite low ( $< 0.03$ ).

The possibility that nutrient levels used in this experiment were not limiting cannot be discounted, although the lowest nutrient level used was 12.5% of the maximum. It is also possible that even though nutrient levels are not important in determining gametophyte gender, they are important to gender allocation within gametophytes. The relative number of antheridia and archegonia in hermaphrodites, or the number of antheridia present in the male may change in response to nutrient levels in the environment. The period of susceptibility to  $A_{CE}$  for undifferentiated gametophytes is between 3 and 6 days (Banks *et al.*, 1993). It is possible that at this young age, the nutrient quality of the environment is not discernable by the gametophyte. Therefore, other indicators of environmental quality, such as light, may be more likely to factor into sex determination.

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